The Second RNA Chaperone, Hfq2, Is Also Required for Survival under Stress and Full Virulence of *Burkholderia cenocepacia* J2315[▽]

Christian G. Ramos, Sílvia A. Sousa, André M. Grilo, Joana R. Feliciano, and Jorge H. Leitão*

IBB—Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering,
Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisbon, Portugal

Received 18 November 2010/Accepted 19 January 2011

Burkholderia cenocepacia J2315 is a highly virulent and epidemic clinical isolate of the *B. cepacia* complex (Bcc), a group of bacteria that have emerged as important pathogens to cystic fibrosis patients. This bacterium, together with all Bcc strains and a few other prokaryotes, is unusual for encoding in its genome two distinct and functional Hfq-like proteins. In this work, we show results indicating that the 188-amino-acid Hfq2 protein is required for the full virulence and stress resistance of *B. cenocepacia* J2315, despite the presence on its genome of the functional 79-amino-acid Hfq protein encoded by the *hfq* gene. Similar to other Hfq proteins, Hfq2 is able to bind RNA. However, Hfq2 is unique in its ability to apparently form trimers *in vitro*. Maximal transcription of *hfq* was observed in *B. cenocepacia* J2315 cells in the early exponential phase of growth. In contrast, *hfq2* transcription reached maximal levels in cells in the stationary phase, depending on the CepR quorum-sensing regulator. These results suggest that tight regulation of the expression of these two RNA chaperones is required to maximize the fitness and virulence of this bacterium. In addition, the ability of Hfq2 to bind DNA, not observed for Hfq, suggests that Hfq2 might play additional roles besides acting as an RNA chaperone.

The RNA chaperone Hfq was initially identified as a protein required for RNA bacteriophage Q_β replication in *Escherichia coli* (13). Subsequently, this mediator of small noncoding RNA (sRNA)-mRNA interactions has gained further relevance due to its role as a master regulator of bacterial metabolism (39).

Much of the knowledge about the roles played by Hfq proteins comes from studies with *Salmonella* (25). Hfq proteins control the translation and decay of several mRNAs (21). The protein forms hexamers that bind preferentially to A/U-rich sequences of RNAs, mediating the interaction of sRNAs with their target mRNAs, thus modifying mRNA translation and/or stability (9, 39).

Several sRNAs that specifically bind to the Hfq protein have been shown to control the translation of their target mRNAs in response to environmental stresses (9). In addition, accumulating evidence indicates that the expression of some virulence genes depends on the presence of a functional Hfq protein. In a recent paper, Chao and Vogel reviewed the roles played by Hfq in several Gram-negative and Gram-positive pathogens. These roles include the control of toxin production, biofilm formation, quorum-sensing regulators, and type III secretion systems, among others (7).

The *Burkholderia cepacia* complex (Bcc) is a group of at least 17 closely related species that emerged in the 1980s as life-threatening and difficult-to-eradicate pathogens among patients suffering from cystic fibrosis (CF) (10). More recently, these pathogens have also been recognized as important

threats to hospitalized patients suffering from other diseases (18, 35).

Several prokaryotes harbor genes encoding two distinct Hfq-like proteins in their genomes. This is the case for the archaeal species *Methanobacterium thermautotrophicum* and *Archaeoglobus fulgidus*, the bacterial species *Bacillus anthracis*, *Magnetospirillum magnetotacticum*, and *Novosphingobium aromaticivorans*, and several species of the *Burkholderia* genus, including all of the members of the Bcc (29).

In a previous work, we performed a functional analysis of the *B. cepacia* *hfq* gene, which encodes a 79-amino-acid protein, and identified the *hfq2* gene, which encodes an unusually long Hfq-like protein composed of 188 amino acid residues (36).

In this report, we describe the functional analysis of the sRNA chaperones Hfq and Hfq2 from the highly virulent clinical isolate *B. cenocepacia* J2315 and compare the transcription patterns of *hfq* and *hfq2* in cells in different growth phases. Results are also presented showing the requirement of both functional RNA chaperones, Hfq and Hfq2, for *B. cenocepacia* J2315 optimal survival of stress and for full virulence.

MATERIALS AND METHODS

Bacterial strains, culture conditions, plasmids, and primers. The bacterial strains and plasmids used in this study are described in Table 1. The primers and oligonucleotides used are listed in Table 2. *B. cenocepacia* strains were maintained on PIA (*Pseudomonas* isolation agar) plates supplemented with 700 μg ml⁻¹ trimethoprim for the *B. cenocepacia* CJ1 mutant or *B. cenocepacia* strains transformed with pMLBAD derivatives. *B. cenocepacia* H111-R (Δ cepR) was maintained on PIA plates supplemented with 50 μg ml⁻¹ kanamycin. *E. coli* strains were maintained on LB (Lennox broth) plates supplemented with antibiotics when appropriate. Liquid cultures were grown at 37°C in LB liquid medium supplemented with antibiotics when appropriate with orbital agitation (250 rpm). Plasmids pMLBAD, pSAS3, and pCGR9 were used in complementation experiments. Plasmid pCGR9 was obtained by ligating to BamHI/SalI-digested pMLBAD the BamHI/SalI-digested 595-bp amplicon obtained by PCR with primers UP-HF2-C and Low-HF2-C (Table 2) using *B. cenocepacia* J2315

* Corresponding author. Mailing address: Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Torre Sul, Piso 6. Av. Rovisco Pais, 1049-001 Lisbon, Portugal. Phone: 351 218417688. Fax: 351 218419199. E-mail: jorgeleitao@ist.utl.pt.

[▽] Published ahead of print on 28 January 2011.

TABLE 1. Bacterial strains and plasmids used in this work

Bacterial strain or plasmid	Genotype or description	Reference or source
Strains		
<i>B. cenocepacia</i> J2315	CF patient sputum isolate	11
<i>B. cenocepacia</i> J2315(pCGR9)	J2315 transformed with pCGR9	This study
<i>B. cenocepacia</i> J2315(pSAS3)	J2315 mutant transformed with pSAS3	This study
<i>B. cenocepacia</i> CJ1 (Δhfq)	J2315 derivative with <i>hfq</i> gene interrupted by <i>tmp</i> gene cassette	This study
<i>B. cenocepacia</i> CJ1(pCGR9)	CJ1 mutant transformed with pCGR9	This study
<i>B. cenocepacia</i> CJ1(pSAS3)	CJ1 mutant transformed with pSAS3	This study
<i>B. cenocepacia</i> CJ2' (<i>hfq2</i> ::Cm)	J2315 derivative with <i>hfq2</i> gene interrupted by chloramphenicol resistance gene cassette	This study
<i>B. cenocepacia</i> CJ2 ($\Delta hfq2$)	CJ2' derivative with <i>hfq2</i> gene interrupted by <i>flp</i> recombinase excision scar without <i>cat</i> gene	This study
<i>B. cenocepacia</i> CJ2(pCGR9)	CJ2 mutant transformed with pCGR9	This study
<i>B. cenocepacia</i> CJ2(pSAS3)	CJ2 mutant transformed with pSAS3	This study
<i>B. cenocepacia</i> CJ3 (<i>hfq</i> ^{sil} $\Delta hfq2$)	CJ2 mutant transformed with pJFR1	This study
<i>B. cenocepacia</i> H111	CF patient sputum isolate	15
<i>B. cenocepacia</i> H111-R ($\Delta cepR$)	<i>cepR</i> ::Km mutant of H111	15
<i>E. coli</i> DH5 α	F ⁻ <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG</i> $\phi 80dlacZ\Delta M15$ $\Delta(lacZYA-argF)U169$ <i>hsdR17</i> (r _K ⁻ m _K ⁺) λ ⁻	20
<i>E. coli</i> OP50	<i>C. elegans</i> feeding strain	5
<i>E. coli</i> Sure	<i>endA1 glnV44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC</i> ::Tn5 <i>uvrC</i> $\epsilon 14^{-}$ $\Delta(mcrCB-hsdSMR-mrr)171$ F' <i>[proAB⁺ lacI⁴ lacZ</i> $\Delta M15$ Tn10]	Stratagene
<i>E. coli</i> BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B</i> (r _B ⁻ m _B ⁻) λ (DE3 [<i>lacI lacUV5-T7 gene 1 ind-1 sam-7 nin-5</i>])	Invitrogen
<i>E. coli</i> MC4100	F ⁻ [<i>araD139</i>] _{B₁₇} $\Delta(argF-lac)169$ λ^{-} $\epsilon 14^{-}$ <i>flhD5301</i> $\Delta(fruK-yeiR)725$ (<i>fruA25</i>) <i>relA1 rpsL150</i> (Str ^r) <i>rbsR22</i> $\Delta(fimB-fimE)632$::IS1) <i>deoC1</i>	6
<i>E. coli</i> GS081	<i>E. coli</i> MC4100 Δhfq	43
Plasmids		
pKD46	Lambda red recombinase expression plasmid, contains 2,154 nucleotides (31088-33241) of phage lambda, including terminator downstream of <i>ftp</i> , <i>exo</i> , tL3, and a small open reading frame	8
pKD3	Template plasmid for gene disruption; resistance gene is flanked by FRT sites	8
pCP20	Contains yeast <i>flp</i> recombinase gene, FLP, chloramphenicol and ampicillin resistance genes, and a temperature-sensitive replicon	8
pWH884	Ap ^r , used for protein expression, N-terminal His tag	32
pMLBAD	pBBR1 <i>ori</i> , <i>araC/P_{BAD}</i> , Tp ^r , <i>mob</i> ⁺	17
pCGR4	pET23a+ with <i>hfq</i> gene cloned, C-terminal His tag	36
pCGR8	pCR 2.1 containing 1.5-kb PCR fragment with <i>hfq-2</i> gene and 450 bp up- and downstream, obtained with primers Hfqgc_up and Hfqgc_low, cloned into XbaI/HindIII sites	This study
pCGR8.1	pCGR8 with PCR fragment obtained from pKD3 with primers CM_FWD and CM_REV blunt end ligated into AscI site	This study
pCGR9	pMLBAD with <i>hfq2</i> gene obtained with primers up_HF2_C and low_HF2_C cloned into BamHI/SalI sites	This study
pSAS2	pDRIVE with <i>hfq</i> gene interrupted by trimethoprim gene cassette	36
pSAS3	pMLBAD with <i>hfq</i> gene	36
pSAS5	pWH884 with <i>hfq2</i> gene obtained with primers HF2_EU and HF2_EL cloned into BamHI/SalI sites	This study
pJRF1	pMLBAD with antisense sequence of <i>hfq</i> gene cloned into XbaI/HindIII sites	This study
pAMG1	pCRII-TOPO with mtvR sRNA cloned downstream of T7 promoter	Ramos et al., unpublished

chromosomal DNA as the template. Plasmid pJRF1 was obtained by subcloning the HindIII-XbaI fragment of the *hfq* gene from pSAS3 and ligating it into the XbaI-HindIII sites of pMLBAD. The resulting plasmid, pJRF1, expresses an antisense RNA, under the control of the P_{BAD} promoter, that is complementary to the *hfq* mRNA, thus silencing *hfq* by induction with arabinose. Plasmids were introduced into *B. cenocepacia* CJ2 by electroporation as previously described (34). Plasmid pSAS2 was used to construct the *B. cenocepacia* CJ1 mutant. Plasmid pSAS5 (Table 1), obtained by ligating the BamHI/SalI-digested PCR amplicon obtained with primers HF2_EU and HF2_EL (Table 2) to the BamHI/SalI sites of pWH884, was used to express Hfq2 as an N-terminally His-tagged derivative.

DNA manipulation techniques. Total DNA was extracted from cells of exponentially growing liquid cultures of *B. cenocepacia* strains using the DNeasy Blood & Tissue kit (Qiagen). Plasmid isolation and purification, DNA hydrolysis with restriction enzymes (Clontech-Takara), electrophoresis in agarose gels, DNA ligation, and *E. coli* transformations were carried out using standard procedures (30). Amplification of the *hfq* or *hfq2* gene of *B. cenocepacia* J2315 in reverse transcription (RT)-PCR experiments was achieved with primers Hfq2cFWD and Hfq2cREV or Hfq_Cu and Hfq_Cd, respectively (Table 2). *B. cenocepacia* CJ2 was generated using the lambda red helper plasmid (8). Briefly, plasmid pCGR8 was obtained by TA ligation to pCR2.1 of the 2.0-kb PCR product obtained with *B. cenocepacia* J2315 chromosomal DNA as the template

TABLE 2. Oligonucleotides and primers used in this work

Name	Sequence (5'-3') ^a	Purpose	Restriction site	Source
Up_HF2_C	<u>AGGATCC</u> ATGGCCAATCCCGCAGA	Complementation experiments	BamHI SalI	This study
Low_HF2_C	TTGTCGAC <u>TT</u> ACTGGCCGTCCG			This study
Hfq2c_FWD	AGGATCCATGGCCAATCCCGCAGA	RT-PCR	BamHI HindIII	This study
Hfq2c_Rev	TAAGCTTCTGGCCGTCCGGCAC			This study
Hfqgc_up	AATCTAGATGAGCCATATGACG	<i>hfq2</i> mutagenesis	XbaI HindIII	This study
Hfqgc_low	TTTAAGCTTCGTCGAGTCCTA			This study
HF2_EU	AAGGATCCATGGCCAATCCCGCAGA	<i>hfq2</i> overexpression	BamHI SalI	This study
HF2_EL	TTGTCGAC <u>TT</u> ACTGGCCGTCCG			This study
Hfq_Cu	<u>GAATTC</u> ATGAGCAACAAAGG	RT-PCR	EcoRI XhoI	
Hfq_Cd	TTTGCATCTCGAGGGGACGA	RT-PCR		
CM_FWD	GTGTAGGCTGGAGCTGCTTC	Amplification of FRT-flanked <i>cat</i> gene from pKD3		(8)
CM_REV	GGACCATGGCTAATTCCTCAT			(8)
HFQ	AAAGGGCAATTGTTACAAG	Northern analysis of <i>hfq</i>		This study
HFQ2	TACGGCTCCCGCGAGCCGCGTGAA	Northern analysis of <i>hfq2</i>		This study
5S	TTCGGGATGGGAAGGGGTGGGA	Northern blot analysis loading control		This study

^a Restriction endonuclease recognition sequences are underlined.

and primers Hfqgc_UP and Hfqgc_LOW (Table 2). Plasmid pCGR8 was digested with SmaI, ethanol precipitated, and suspended in elution buffer (10 mM Tris, pH 8.0). Plasmid pKD3 (8) was used as a PCR template to obtain the chloramphenicol acetyltransferase (CAT) gene cassette with primers CM FWD and CM REV (Table 2) flanked by the FLP recombinase recognition site as previously described (8). The CAT resistance cassette was gel purified, filled in with the Klenow large fragment of DNA polymerase I (Invitrogen), and blunt end ligated to the digested pCGR8 plasmid, yielding pCGR8.1. All plasmid constructions were confirmed by nucleotide sequencing.

RT assays were performed using the Superscript one-step RT-PCR kit (Invitrogen) with appropriate gene-specific primers (Table 2).

Generation of mutants and confirmation of insertional inactivation by PCR. Plasmid pCGR8.1 was digested with EcoRI, and the 1.7-kb fragment corresponding to the 900-bp CAT element, the 580-bp *hfq2* gene, and the 220-bp *hfq2* gene surrounding region was purified after agarose gel electrophoresis and resuspended in elution buffer (10 mM Tris, pH 8.0). Cells of *B. cenocepacia* J2315 transformed with lambda red helper plasmid pKD46 were grown at 30°C to an optical density at 600 nm (OD₆₀₀) of ~0.6 in 5-ml SOB (30) liquid cultures with 1.5 mg ml⁻¹ ampicillin and 1% (wt/vol) L-arabinose. Cells were made electrocompetent and electrotransformed as previously described (22, 36) using 25-μl cell suspensions and 1 to 10 μg of the 1.7-kb digestion product from pCGR8.1. After electroporation, 1 ml of SOC (30) liquid medium was added to the cell suspension, followed by incubation for 4 h at 37°C. The cell suspension was then spread onto the surfaces of PIA plates supplemented with 250 μg ml⁻¹ chloramphenicol. Cm^r transformants were made electrocompetent and transformed with plasmid pCP20. The resulting cell suspensions were incubated at 30°C for 4 h and then spread onto the surface of nonselective PIA and incubated overnight at 42°C for plasmid loss. Colonies were replica plated on PIA plates not supplemented or supplemented with 250 μg ml⁻¹ chloramphenicol. Colonies unable to grow in the presence of chloramphenicol were kept for further analysis.

To confirm the insertional inactivation of *hfq2* in *B. cenocepacia* J2315, two colony PCR experiments were performed. Primers Hfq2cFWD and Hfq2cREV were used to confirm the occurrence of a double-crossover recombination in the *hfq2* gene (with an expected size of 1,480 bp), while primers CM_FWD and CM_REV were used to confirm the loss of the CAT element from the chromosome of the *B. cenocepacia* *hfq2* insertional mutant. Additionally, the *hfq2* mutation and the *cat* excision were confirmed by nucleotide sequencing of the PCR product obtained with primers Hfq2cFWD and Hfq2cREV and the chromosomal DNA from the *B. cenocepacia* *hfq2* insertional mutant as the template. The deletion mutant was named CJ2. The *B. cenocepacia* J2315 *hfq* insertion mutant

(*B. cenocepacia* CJ1) was generated using pSAS2 (36) based on previously described methods (36).

A *Δhfq2* mutant with the *hfq* gene silenced (*hfq*^{sil}) was constructed by cloning the *hfq* PCR product digested with XbaI/HindIII into the XbaI/HindIII sites of pMLBAD, yielding pJRF1, which, upon induction with arabinose, expresses part of the antisense transcript of *hfq*, thus blocking *hfq* mRNA translation. pJRF1 was electroporated into *B. cenocepacia* CJ2, and transformants were selected on PIA plates supplemented with 700 μg ml⁻¹ trimethoprim. This conditional mutant strain (*hfq*^{sil} *Δhfq2*) was named *B. cenocepacia* CJ3.

Cloning, overexpression, and purification of the *hfq* and *hfq2* gene products.

The Hfq protein was produced as a C-terminally His-tagged derivative expressed in *E. coli* BL21(DE3) cells harboring pCGR4 (Table 1) based on previously described methodologies (36). Purified, His-tagged Hfq in hexameric form (Hfq₆) was dialyzed with 25 mM Tris-Cl (pH 7.5)–150 mM NaCl–0.5 mM EDTA (17).

The Hfq2 protein was overexpressed as a recombinant protein with six additional histidine residues at its N terminus in *E. coli* Sure cells harboring pSAS5 (33, 40). After purification by affinity chromatography, fractions containing His-tagged Hfq2 were dialyzed overnight against Hfq storage buffer (36) or 20 mM phosphate buffer (pH 8.0) using a Slide-A-Lyzer cassette (Pierce) with a 10-kDa cutoff. Purified proteins (as judged by Coomassie staining of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis [PAGE] gel) were analyzed by Western blotting based on the methods of Sousa et al. (36) using the Penta-His HRP Conjugate antibody (Qiagen) and TMB (Sigma) as the substrate.

RNA extraction and purification. Total RNA was obtained from cells of *B. cenocepacia* strains J2315, CJ1, CJ2, H111, and H111-R harvested by centrifugation at appropriate time points using the Ribopure RNA isolation kit (Ambion). RNA concentration was estimated in an ND 1000 spectrophotometer (Nanodrop). RNA quality was assessed by visual inspection after electrophoresis in agarose/formaldehyde denaturing gels.

Electrophoretic mobility shift assays (EMSAs). The 136-nucleotide small non-coding RNA mtvR (C. G. Ramos, A. M. Grilo, and J. H. Leitão, unpublished data) was used in EMSAs. For this purpose, mtvR was transcribed *in vitro* from the pAMG1 plasmid using the MegaShortScript kit (Ambion), purified from a 7 M urea–8% polyacrylamide gel, and 5' end labeled with fluorescein isothiocyanate (FITC)-12-UTP (Amersham) using T4 polynucleotide kinase (NEB). Samples were prepared in a total volume of 25 μl of RNA binding buffer (36) containing 0, 5, 25, or 50 pmol of purified, His-tagged Hfq2 or 0, 0.1, 1, 5, 25, or 50 mM Hfq and 10 pmol of FITC-labeled mtvR sRNA. Nonlabeled yeast tRNA (Ambion) was added in excess to each sample to minimize nonspecific binding.

After incubation for 15 min at 30°C, the mixtures were loaded onto a 10% polyacrylamide gel in 1× Tris-borate-EDTA (TBE) buffer containing 1% (vol/vol) glycerol. Electrophoresis was performed at 4°C and 150 V for 2 h. RNAs were electroblotted at 200 mA for 1 h onto a BrightStar-Plus (Ambion) membrane soaked in 0.5× TBE and UV cross-linked. RNAs were detected by chemiluminescence using alkaline phosphatase-conjugated anti-FITC antibody (Amersham) in combination with the CDP-Star alkaline phosphatase substrate (Novagen).

Discontinuous native protein gel electrophoresis and circular dichroism (CD) experiments. Discontinuous native protein gel electrophoresis of purified, His-tagged Hfq2 was performed based on previously described methods (37). Briefly, aliquots of 20-μl protein samples (6 μg) were added to 5 μl of loading buffer (100 mM Tris-Cl [pH 8.0], 40% glycerol, 0.5% Brilliant Blue G) and incubated for 10 min at room temperature. To confirm the molecular mass of the Hfq2 oligomer, 20-μg samples of the proteins aldolase (158 kDa), bovine serum albumin (66 and 132 kDa), and ovalbumin (44 kDa) were used as molecular mass standards. The samples were visualized after 8% (wt/vol) discontinuous native PAGE (30) at 4°C and 100 V for 2 h.

CD spectroscopy experiments were performed using 36 μg of purified, His-tagged Hfq2 in 20 mM phosphate buffer (pH 8.0) and processed as previously described (3). The far UV CD spectrum was recorded at 25°C in a cell with a 1-mm path length using a PiStar-180 spectrometer (Applied Photophysics) at a scan speed of 60 nm/min, a time constant of 1 s, and a bandwidth of 2 nm. The neural network analysis method was used to estimate the Hfq2 protein secondary structure from the far UV CD spectrum data (1). Results are the means of at least 10 wavelength scans.

Northern analysis. The levels of *hfq* or *hfq2* mRNA were assessed by Northern blot analysis using 2 μg of total RNA purified from cells harvested from cultures in different growth phases. Samples containing 2 μg of total RNA were loaded into an 8 M urea–6% polyacrylamide gel and separated by electrophoresis in 1× TBE at a constant current of 35 mA. After electrophoresis, total RNA was electrotransferred to a Hybond-N⁺ membrane (Amersham) at 20 V for 16 h at 4°C using 0.5× TBE.

Oligonucleotide probes (Table 2) were FITC labeled using the Fluorescein-High Prime labeling kit (Roche) and purified using the Quick-Spin column for labeled DNA purification (Roche). Prehybridization and hybridization were performed as previously described (14, 16). The 5S RNA was used as a loading control in all Northern blot experiments.

Hybridization signals were detected using a Typhoon 8600 variable-mode imager scanner (Amersham Biosciences Europe) after selecting the fluorescein emission laser, blue filter, 500-V excitation, and 200-μm resolution. Relative mRNA abundance analysis was estimated with the UN-SCAN-IT gel software suite (Silk Scientific, Inc.) using the band intensities of the 5S RNA as a reference. Affinity constants (K_D) were calculated by assuming a single binding site and using the equation $K_D = [\text{protein-bound RNA}]/[\text{total RNA}]$.

DNA binding experiments. The ability of Hfq2 to bind DNA was assessed using gel shift assays as previously described (28). For this purpose, 100 fmol of the FITC-labeled 124-bp DNA fragment (corresponding to part of the *araC* promoter and coding region) was obtained by restriction of plasmid pMLBAD with EcoRV and ClaI, followed by agarose gel electrophoresis, band excision, and purification with the NucleoSpin Extract II kit (Macherey-Nagel). The purified DNA fragments were incubated for 30 min at 25°C with His-tagged Hfq or Hfq2 (0.2, 1, and 2 μM) in a total volume of 20 μl of DNA binding buffer (28) supplemented with 1 mM ATP and 100 nmol of yeast tRNA (Ambion). Control experiments were performed using the DNA probe and 500 pM Pbr (28). All mixtures contained 10 nmol of the unlabeled 322-bp fragment obtained by pUC19 restriction with PvuII as competing nonlabeled DNA. After incubation, the reaction products were subjected to native 10% PAGE at 4°C and 150 V for 3 h. Retardation corresponding to DNA-protein complexes was detected using a Typhoon 8600 variable-mode imager scanner with the fluorescein emission laser, blue filter, 650-V excitation, and 200-μm resolution (Amersham Biosciences Europe). The affinity constant (K_D) was calculated by assuming a single binding site and using the equation $K_D = [\text{protein-bound DNA}]/[\text{total DNA}]$.

Stress susceptibility experiments. The susceptibility of strains of *B. cenocepacia* (J2315, J2315 with plasmid pSAS3 or pCGR9, the CJ1 mutant, CJ1 with plasmid pSAS3 or pCGR9, the CJ2 mutant, CJ2 with plasmid pCGR9 or pSAS3, and the CJ3 mutant) and *E. coli* (MC4100, GS081, and GS081 with plasmid pSAS3 or pCGR9) to the stresses imposed by growth in solid LB medium containing 1% (wt/vol) L-arabinose and supplemented with 3% (wt/vol) NaCl, 150 μM methyl viologen (pH 5.0; [obtained with 100 mM phosphate-buffered LB]), or growth at 42°C were performed as previously described (28). Results are the means of at least five independent experiments.

Nematode infection experiments. Nematode killing assays and nematode digestive tract bacterial colonization experiments were performed based on previously described methods (36) using *Caenorhabditis elegans* mutant strain DH26. Reported values are the means of triplicate results of at least three independent experiments using a nematode population of $1,250 \pm 25$ worms. Nematodes were photographed at 24, 48, and 72 h postinfection (p.i.) to visually evaluate the condition of their health (magnification shown is ×40).

Nucleotide and amino acid sequence analysis and structure predictions. Nucleotide and amino acid sequences were analyzed using bioinformatic tools available at the National Center for Biotechnology Information, the ExPaSy Prosite, or the European Bioinformatics Institute. The Virtual Footprint suite was used for promoter analysis (24) using *Pseudomonas aeruginosa* promoter sequences.

Statistical analysis. All of the data presented were subjected to a one-way analysis of variance of mean values. Error bars represent the standard deviation of the mean.

RESULTS

Two distinct *hfq*-like genes are found in *B. cenocepacia* J2315 chromosome I. In a previous work, we performed a functional analysis of the 79-amino-acid protein Hfq (BCAL1879) in the genome sequence of *B. cenocepacia* J2315, Fig. 1A) from the clinical isolate *B. cepacia* IST408 and described the identification of a second 188-amino-acid putative Hfq-like protein in *B. cenocepacia* J2315, Hfq2 (36), which was the focus of this work. The gene encoding Hfq2 (BCAL1538) is also located in chromosome I, about 370 kb from the gene for Hfq (Fig. 1B), and is flanked by genes encoding predicted membrane proteins, exported proteins, regulatory proteins, and an acyl coenzyme A (CoA) synthetase (Fig. 1B).

Inspection of the complete genomes of other members of the Bcc revealed that all encode proteins highly similar to Hfq and Hfq2. Moreover, these Hfq-like protein-encoding genes are located in similar loci (data not shown).

Construction of *hfq* and *hfq2* *B. cenocepacia* J2315 mutants. In order to gain insights into the biological functions of this second Hfq-like protein, two mutant strains were constructed, CJ2' and CJ2. CJ2' was derived from *B. cenocepacia* J2315 by the insertion of a *cat* gene flanked by FRT sites. CJ2 was obtained by excision of the *cat* gene from CJ2', introducing three stop codons after nucleotide position 211, thus inactivating the *hfq2* gene. The *B. cenocepacia* CJ1 mutant was also constructed from *B. cenocepacia* J2315 by insertional inactivation of the *hfq* gene with a trimethoprim resistance cassette (Fig. 1A and C) using plasmid pSAS2 and based on previously described methodologies (36). Despite several attempts to construct a $\Delta hfq \Delta hfq2$ double mutant, we were not successful. Therefore, a conditional mutant with the *hfq* gene silenced (*hfq*^{sil}) was obtained by introducing pJRF1 into $\Delta hfq2$ mutant strain CJ2, yielding *hfq*^{sil} $\Delta hfq2$ mutant strain CJ3. Upon induction with arabinose, an *hfq* antisense RNA is expressed from plasmid pJRF1, thus silencing *hfq* in the $\Delta hfq2$ background. In order to confirm that the *hfq* and *hfq2* genes were not expressed in the CJ1 and CJ2' mutants, respectively, total RNA was extracted from cells of wild-type strain *B. cenocepacia* J2315 and of the mutants CJ1 and CJ2' (before excision of the *cat* gene) and used in RT experiments. No amplification bands corresponding to the *hfq* or *hfq2* mRNA could be detected for the *B. cenocepacia* CJ1 and CJ2' mutants, respectively, thus confirming the inactivation of the *hfq* and *hfq2* genes (Fig. 2A). The CJ2 mutant was used thereafter to avoid effects due to the use of chloramphenicol.

FIG. 1. Genetic organization of the *hfq* and *hfq2* genes in *B. cenocepacia* J2315. (A) Genetic organization of the *hfq* gene locus showing the trimethoprim (Tmp) cassette insertion. (B) Genetic organization of the *hfq2* gene locus. Open reading frames BCAL1535 (membrane protein with unknown function), BCAL1536 (σ^{54} -dependent transcriptional regulator), BCAL1537 (putatively exported lipoprotein), BCAL1538 (Hfq2 chaperone), BCAL1539 (putative exported protein), BCAL1540 (transmembrane lipoprotein), BCAL1541 (acyl-CoA synthetase), BCAL1542 (TetR family regulatory protein), and BCAL1543 (major facilitator superfamily protein) are represented in scale. The positions of the three nonsense stop signals introduced after nucleotide 211 are indicated by the filled flag (*hfq2::Frt*). Primers used in RT-PCR experiments are indicated by the letters F (Hfq2c_Fwd) and R (Hfq2c_Rev). The predicted *CepR*-dependent promoter is also indicated. (C) PCR amplification of the *hfq* and *hfq2* genes from *B. cenocepacia* wild-type (WT) strain J2315 and mutant strains CJ1 (Δhfq) and CJ2 ($\Delta hfq2$).

The *hfq* and *hfq2* genes are differentially expressed. In order to gain further clues to the regulation of both the *hfq* and the *hfq2* genes, we investigated their mRNA levels in *B. cenocepacia* strain J2315. The levels of mRNA corresponding to *hfq2* increased about 2-fold, reaching maximal values in cells in the stationary phase of growth (Fig. 2B). In contrast, the levels of mRNA corresponding to *hfq* were maximal in cells in the early exponential phase of growth (Fig. 2B), decreasing almost 2.5-fold in cells in the stationary phase (Fig. 2C).

The mRNA levels of *hfq2*, but not *hfq*, are quorum sensing dependent. A bioinformatic search for putative promoter sequences upstream of the *hfq2* gene revealed a putative *lux* box-like consensus sequence located 50 bp upstream of the *hfq2* gene ATG codon (Fig. 1B). In *B. cenocepacia*, *CepR* is a LuxR family transcription regulator which binds to *lux* box sequences (42). Therefore, we used total RNA extracted from the well-studied *B. cenocepacia* isolate H111 and its derivative *cepR* mutant in Northern blot experiments to assess the mRNA levels of *hfq2* in a *cepR*-defective mutant. As shown in Fig. 2C, the band corresponding to the *hfq2* mRNA is significantly weaker in the *cepR* mutant than in the *B. cenocepacia* H111 wild-type strain and is only detected at 24 h of growth.

The Hfq2 C terminus contains a repeated motif. Compared to the 79-amino-acid protein Hfq, which is 100% identical to the *B. cepacia* IST408 Hfq protein previously characterized by our research group (36), the *B. cenocepacia* J2315 Hfq2 protein is 109 amino acid residues longer (with a total of 188 residues) (Fig. 3A). Alignment of the *B. cenocepacia* J2315 Hfq and Hfq2 proteins and with other large Hfq proteins revealed that

the two proteins share a significant level of conservation between residues 10 and 65, with 60% identity and 75% similarity (Fig. 3B). The typical conserved core motifs of Hfq chaperones were also highly conserved in Hfq2, namely, the Sm1 and Sm2 motifs, structurally conserved loops 3 and 5, and RNA binding residues ¹⁰Gln, ⁶²Ile, and ⁶³Ser (Fig. 3A). The ⁸His, ⁹Pro, ¹⁰Gln, ¹¹Asn, ⁶²Ile, ⁶³Ser, ⁶⁴Thr, ⁶⁵Ile, and ⁶⁶Gln residues in Hfq2, which are predicted to be involved in substrate binding, were conserved only in Hfq proteins from *Burkholderia* strains (Fig. 3A).

Interestingly, the C-terminal extension of Hfq2 and the Hfq homologues from *B. cenocepacia* MCO-3, *B. vietnamensis* G4, and *B. multivorans* ATCC 17616 were all composed of a glycine-rich region (⁷⁰G to ¹⁷³G), a putative DNA binding domain (⁸⁹G to ¹²⁹E), and the repetitive motif RE(P/S)RRXX(E/G)(G/S), which occurs five times in Hfq2, as highlighted in Fig. 3A.

Hfq2 forms trimers *in vitro*. Secondary-structure predictions indicate that 6% of the amino acid residues of Hfq2 fold into an α -helix and 31% fold into a β -sheet. In loose agreement with these predictions, calculations performed based on CD spectroscopy results indicate that *B. cenocepacia* Hfq2 is composed of 5% α -helix and 47% β -sheet (Fig. 4A). This discrepancy probably derives from the fact that the computational structure prediction of *B. cenocepacia* J2315 Hfq2 was based on the 69-amino-acid crystal structure of the *E. coli* Hfq protein.

The ability of purified, His-tagged Hfq2 (Fig. 4B) to form multimeric structures was investigated by discontinuous native PAGE. A major band with approximately three times the mo-

FIG. 2. Expression analysis of the *hfq* and *hfq2* genes. (A) RT of *hfq* and *hfq2* RNA samples obtained from cells of *B. cenocepacia* wild-type (WT) strain J2315 and the CJ1 (Δhfq) and CJ2' ($\Delta hfq2$) mutants. Control experiments were carried out using chromosomal DNA. (B) Northern blot analysis of mRNA corresponding to *hfq* and *hfq2* in *B. cenocepacia* J2315. Relative abundance was quantified for both *hfq* (black bars) and *hfq2* (white bars) in *B. cenocepacia* J2315 using the RNA levels of the 5S rRNA as a control. Error bars represent the standard deviations of the means. (C) Detection of *hfq* and *hfq2* mRNA levels in *B. cenocepacia* wild-type strain H111 and a *cepR* mutant strain by Northern blotting. Quantification of *hfq* mRNA in the *B. cenocepacia* wild-type H111 (black bars) and $\Delta cepR$ mutant strain (white bars) and of *hfq2* transcripts in the *B. cenocepacia* wild-type H111 (gray bars) and $\Delta cepR$ mutant (dashed bars) strains was performed using the 5S rRNA mRNA levels as a control. All experiments were repeated at least three times.

lecular mass of monomeric Hfq2 suggests that the protein might exist as a trimer in its active/native form *in vitro*. This result contrasts with the findings for other Hfq proteins and the Hfq protein of *B. cenocepacia* J2315, which forms hexamers *in vitro* (36). A faint band, highlighted in Fig. 4B with an asterisk, with an apparent molecular mass compatible with a tetrameric form of Hfq2 was also observed. Nevertheless, the trimeric form is predominant.

***B. cenocepacia* J2315 Hfq and Hfq2 bind to the sRNA mtvR.** Since both Hfq and Hfq2 possess all of the motifs required for RNA binding, we tested their ability to bind the mtvR sRNA by using EMSAs. Results shown in Fig. 5 clearly show that both proteins are able to bind this sRNA. Interestingly, Hfq seems to bind mtvR more efficiently (60-fold lower K_D) than Hfq2 does.

Hfq2, but not Hfq, binds DNA. Since a DNA binding domain spanning amino acid residues 87 to 128 was bioinformatically predicted for Hfq2, we performed DNA binding assays. For this purpose, we used either purified, His-tagged Hfq or Hfq2 and the 124-bp EcoRV/ClaI DNA fragment of the *araC* promoter of pMLBAD (17). The purified, His-tagged Pbr regula-

tory protein, which is involved in the regulation of *B. cenocepacia* K56-2 phenazine biosynthesis and cellular processes related to stress resistance and virulence (28), was used as a positive control. Results presented in Fig. 6 clearly show that Hfq2 is able to bind DNA, consistent with the predicted occurrence of a DNA binding domain. An apparent binding constant of 92 nM for the Pbr regulator to the DNA fragment was estimated, while a value of 44.2 μ M was estimated for the binding of Hfq2 to this particular DNA fragment. Similar experiments performed using Hfq instead of Hfq2 did not reveal the binding of Hfq to this DNA fragment (data not shown). The biological significance of the ability of Hfq2 to bind DNA is unknown, although we speculate that the protein might act as a transcription regulator.

Hfq2 is required for resistance to stress. We compared the abilities of wild-type *B. cenocepacia* J2315 and its derivative mutants CJ1 and CJ2 (carrying nonfunctional *hfq* and *hfq2* genes, respectively) to survive stress conditions mimicking those faced by the bacterium when colonizing/infecting the CF lung, namely, growth at 42°C, oxidative stress conditions im-

FIG. 3. Hfq2 is highly conserved within the Bcc. (A) Alignment of the *B. cenocepacia* J2315 Hfq and Hfq2 proteins with other Hfq-like proteins showing the predicted secondary structure of Hfq2 above the amino acid residues. The Sm1 and Sm2 motifs are indicated. RNA binding domains are represented by blue boxes. Residues also predicted to be involved in RNA binding are highlighted in yellow. The DNA binding domain is within the gray box. Boxed sequences denote the identified repeats. Identical amino acid residues are marked with an asterisk, and conserved and semiconserved substitutions are marked with double and single dots, respectively. Abbreviations: Bcen, *B. cenocepacia*; Bviet, *B. vietnamiensis*; Bmul, *B. multivorans*; Nmening, *Neisseria meningitidis*; Mcatar, *Moraxella catarrhalis*. (B) Superimposition of the monomeric predicted three-dimensional structures of Hfq and Hfq2 from *B. cenocepacia* J2315. The structural alignment is highlighted in yellow, with a root mean square deviation of 0.910 Å.

posed by methyl viologen, acidic pH, and osmotic stress induced by the addition of 3% NaCl to the culture medium. The CJ3 (*hfq*^{sil} Δ *hfq2*) strain was also included in this analysis. CJ3 carries a nonfunctional *hfq2* gene, and upon induction with L-arabinose, it expresses an antisense RNA targeting *hfq*. In comparison with wild-type strain J2315 overexpressing the *hfq*

or *hfq2* gene, the results clearly indicate that mutation of the *hfq* or the *hfq2* gene rendered the respective strain CJ1 or CJ2 more susceptible to the tested stress. This indicates that both genes are required for optimal survival of various stresses (Fig. 7). The increased susceptibility of these mutants was at least partially rescued by the ectopic expression of *hfq* or *hfq2*. This

FIG. 4. Hfq2 forms trimeric structures *in vitro*. (A) Far UV CD spectrum of Hfq2 from *B. cenocepacia* J2315. The regions of the spectrum corresponding to the α -helix and β -sheet are indicated. (B) Discontinuous native PAGE showing a predominant Hfq2 band at ~ 63 kDa compatible with a trimeric form of the protein. The monomeric form is also visible. Possible higher-order complexes (marked by an asterisk) can be seen. BSA, bovine serum albumin.

FIG. 5. *B. cenocepacia* J2315 Hfq2 is able to bind sRNAs. (A) EMSA experiments using 0, 0.2, 1, and 2 μ M purified, His-tagged $[Hfq2]_3$ and 400 nM FITC-12-UTP 5'-end-labeled *mtvR* sRNA. (B) With increasing $[Hfq]_6$, additional Hfq multimers bind to the *mtvR* sRNA. $[Hfq2]_3$, trimeric form of Hfq2; $[Hfq]_6$, hexameric form of Hfq; R-H, complexes formed by $[Hfq2]_3$ and the *mtvR* sRNA; R-H, complexes formed by $[Hfq]_6$ and the *mtvR* sRNA (R).

partial complementation has been observed before (28) and is derived from insufficient amounts of L-arabinose to fully induce the P_{BAD} promoter. Remarkably, the CJ3 strain exhibited the highest susceptibility to the stresses studied, reinforcing the observation that both the *hfq* and *hfq2* genes are required for optimal survival of *B. cenocepacia* J2315 under stress conditions. Overexpression of *hfq* rendered *B. cenocepacia* wild-type strain J2315 more resistant to methyl viologen and NaCl, while overexpression of *hfq2* rendered the wild-type strain more resistant to growth at 42°C and pH 5.0. Both the *hfq* and *hfq2* genes of *B. cenocepacia* J2315 were used in complementation experiments with *E. coli hfq* mutant strain GS081. Results shown in Fig. 8 indicate that both genes are functional in *E. coli*, being able to partially rescue the observed growth defects of the *E. coli hfq* mutant when it is exposed to oxidative stress imposed by methyl viologen. It is worth noting that *hfq2* was able to fully complement the ability of the *E. coli hfq* mutant to grow at 42°C or pH 5.0. Remarkably, when *hfq2* was provided in *trans* to the *E. coli hfq* mutant, this strain became less susceptible to the osmotic stress imposed by 3% NaCl, compared with *E. coli* wild-type strain MC4100.

***hfq* and *hfq2* are both required for full virulence of *B. cenocepacia* J2315.** The role played by *hfq* and *hfq2* in *B. cenocepacia* J2315 virulence using *C. elegans* as an infection model was investigated based on slow-killing assays (36). The abilities of wild-type *B. cenocepacia* J2315, *hfq* mutant strain CJ1, *hfq2* mutant CJ2 strain, and the CJ3 antisense knockdown strain, as well as the wild-type strain overexpressing *hfq* or *hfq2* in *trans*, to kill *C. elegans* larvae at the L4 stage were compared.

Results shown in Fig. 9A clearly indicate that overexpression of *hfq* rendered the wild-type strain more virulent. This was also observed, although to a lesser extent, until the second day of infection by the wild-type strain overexpressing *hfq2*. Compared to the wild-type strain, the CJ3 strain was significantly attenuated, and this effect was particularly evident at 3 or 4 days p.i. Figure 9 also clearly indicates that mutations in *hfq* (panel B) or *hfq2* (panel C) significantly reduced the virulence of *B. cenocepacia* J2315. Although general overexpression of

FIG. 6. *B. cenocepacia* J2315 Hfq2 is able to bind DNA. The ability of Hfq2 to bind DNA was evaluated by band shift assays. The Pbr transcription regulator (28) was used as a positive control. All binding kinetic parameters were calculated by assuming a fixed-term single-site binding isotherm.

hfq or *hfq2* by the CJ1 or CJ2 mutant led to numbers of surviving worms comparable to or below those of worms surviving infection by the wild-type strain, a reduction in survival was observed when worms were challenged with the CJ2 mutant overexpressing *hfq*.

The total number of CFU surviving within the nematode's digestive tract was consistent with the relative virulence of the wild-type strain, the CJ1 and CJ2 mutants, and the complemented CJ1 and CJ2 mutants (Fig. 9D). Visual inspection of worms infected at 24, 48, or 72 h p.i. with *B. cenocepacia* J2315, the CJ2 mutant, or the complemented CJ2 mutant showed that worms infected with the CJ2 mutant appeared healthier, in agreement with the requirement of a functional *hfq2* gene for the full virulence of *B. cenocepacia* J2315 (panel E).

DISCUSSION

Bcc members are among a small number of prokaryotes that encode two distinct copies (highly conserved among Bcc members [27]) of Hfq-like proteins in their genomes, and to the best of our knowledge, this is the first study of a bacterium harboring two distinct and functional Hfq-like proteins.

The similarity found between Hfq2 and the Sm1 motif of the Sm and Lsm proteins from eukaryotes and archaea suggests that these bacterial proteins derive from an ancestral Sm protein (29). In the case of archaea, it was proposed that the ancestral Sm-like protein was subjected to several duplication events, producing the present related Sm-like proteins in yeast, humans, and other eukaryotes (29). Since no significant homology of the C-terminal extension of Hfq2 could be found within other bacterial genome sequence databases besides Bcc, and considering the limited number of bacteria with two distinct Hfq-like genes, we hypothesize that both *hfq* and *hfq2* have arisen in the genomes of Bcc through the duplication of an ancestor gene, followed by evolutionary events that led to two distinct and functional Hfq-like proteins in the current Bcc genomes.

A recent analysis of the genome sequence of *B. cenocepacia* J2315 suggested that this bacterium has recently adapted to the

FIG. 7. Both Hfq and Hfq2 are required for stress resistance. The susceptibility of the indicated *B. cenocepacia* strains to various stresses was tested by spot inoculating serially diluted bacterial suspensions with an initial OD₆₄₀ of 1.0. wt, wild type.

ecological niche that is the human host, evidenced by a genome well equipped with functions associated with virulence in the CF lung, where the organism must face adverse conditions, including oxidative stress conditions associated with survival within macrophages (12). Our results show that both Hfq-like proteins are required for and contribute to the bacterium’s survival of stresses related to the colonization and infection of

the CF host, particularly evidenced by the phenotypes of stress susceptibility observed in the CJ3 strain, which harbors a non-functional *hfq2* gene and expresses an antisense RNA targeting *hfq*. This strain was constructed because we were unable to generate a stable *Δhfq Δhfq2* double mutant. Interestingly, results also show that overexpression of *hfq* in the wild-type strain led to increased resistance to pH 5.0 and

FIG. 8. *B. cenocepacia* J2315 Hfq and Hfq2 are able to complement an *E. coli* *hfq* mutation. The ability of the *B. cenocepacia* *hfq* or *hfq2* gene to complement the *hfq* mutation in *E. coli* GS081 was evaluated by subjecting the indicated *E. coli* strains to the stresses indicated. *phfq*, *E. coli* GS081 transformed with pSAS3; *phfq2*, *E. coli* GS081 transformed with pCGR9; wt, wild type.

FIG. 9. Hfq and Hfq2 are both required for full *B. cenocepacia* J2315 virulence in the nematode *C. elegans*. (A) Abilities of *B. cenocepacia* wild-type strain J2315 (wt, black bars), J2315 harboring pSA3 (wt + *phfq*, dashed bars) or pCGR9 (wt + *phfq2*, squared bars), and CJ3 (*hfq*^{sil} *Δhfq2*, dotted bars) to kill the nematode *C. elegans* DH26. (B) Abilities of *B. cenocepacia* *hfq* mutant CJ1 (*Δhfq*, gray bars) and CJ1 harboring pSA3 (*Δhfq* + *phfq*, dashed gray bars) or pCGR9 (*Δhfq* + *phfq2*, squared gray bars) to kill the nematode *C. elegans* DH26. (C) Abilities of the *B. cenocepacia* *hfq2* mutant CJ2 (*Δhfq2*, white bars) and CJ2 harboring pSA3 (*Δhfq2* + *phfq*, dashed bars) or pCGR9 (*Δhfq2* + *phfq2*, squared bars) to kill the nematode *C. elegans* DH26. (D) Abilities of *B. cenocepacia* J2315 (wt, black bars), CJ1 (*Δhfq*, gray bars), CJ2 (*Δhfq2*, white bars), CJ1 harboring pSA3 (*Δhfq* + *phfq*, dashed gray bars), and CJ2 harboring pCGR9 (*Δhfq2* + *phfq2*, squared bars) to colonize the nematode's digestive tract, expressed as the number of CFU/worm. (E) The health status of worms after infection for 24, 48, or 72 h with the indicated strains was assessed by visual inspection. Worms in panel E were randomly chosen and photographed.

3% NaCl, while the wild-type strain overexpressing *hfq2* exhibited increased resistance to thermal stress and to 150 μ M methyl viologen. These results suggest that, besides a common role, as evidenced by the ability of both Hfq and Hfq2 to complement an *E. coli* *hfq* mutant used in this study, each protein might play additional roles related to specific stresses. This specificity possibly derives from the different C termini of Hfq and Hfq2.

Large Hfq proteins with an extended C terminus have been described in some bacterial species of the beta- and gamma-proteobacteria. Glycine-rich domains between the N and C termini (31) have been described for the few large Hfq proteins characterized so far. *B. cenocepacia* Hfq2 also contains a glycine-rich domain and a repetitive pattern that occurs five times in its C terminus (27), although no biological function has yet been assigned. A repetitive but distinct pattern was described for *Acinetobacter baylyi* Hfq which was also found in DnaJ-like chaperones (41), but no functions were attributed to these features of the protein. The biological function of the C terminus of the 210-amino-acid Hfq protein from *Moraxella catarrhalis* is also unknown (2).

In the case of *E. coli*, Vecerek et al. (40) have shown that truncated variants of Hfq lacking the C-terminal extension, effectively bind sRNAs but are defective in the establishment of sRNA-mRNA base pairing, as well as in Hfq autoregulation, suggesting that the C terminus constitutes an unrecognized

RNA interaction surface with specificity for mRNAs (40). Although *E. coli* Hfq and *B. cenocepacia* Hfq2 are quite distinct, we cannot exclude the possibility that the *B. cenocepacia* J2315 Hfq2 C terminus plays roles similar to those of the *E. coli* Hfq C terminus. A putative DNA binding domain was also identified in the C-terminal extension of Hfq2. Although we present results clearly showing that Hfq2 is able to bind DNA, both the specificity of the interaction and its biological significance remain unknown. Curiously, a recent report on the ability of *E. coli* Hfq to bind DNA highlighted the importance of the C terminus in DNA displacement, especially for residues beyond position 75 (38). This amino acid stretch is absent from the *B. cenocepacia* J2315 Hfq protein.

Results of structure prediction and CD spectroscopy experiments indicate that the Hfq2 glycine-rich C terminus is predominantly unstructured. We anticipate that this disordered region contributes to the difficulty in assembling the protein into a stable multimeric form greater than three monomers. It is worth noting that this contrasts with the hexameric structures of all of the bacterial small Hfq proteins studied so far (4, 19, 26). Moreover, a recent report by Moskaleva et al. (23) highlights the important role played by histidine residue 57 in the establishment of a stable hexameric structure. Curiously, in *B. cenocepacia* Hfq2, ⁵⁷H is replaced by ⁵⁷R, which, together with steric constraints, may account for its inability to form hexamers.

Given the distinct pattern of regulation of expression observed for the two *hfq*-like genes of *B. cenocepacia* J2315, with the RNA levels of *hfq* maximal in cells in the early exponential phase of growth and the levels of *hfq2* RNA being maximal in the stationary phase of growth, it is expected that Hfq and Hfq2 levels must be strictly regulated in the bacterial cell, with this strictly balanced level of the two proteins being required for optimal survival of stress and efficient virulence. Interestingly, our data point out that quorum sensing triggers the expression of *hfq2*, allowing its maximal expression when *hfq* mRNA levels are minimal. The differential regulation of *hfq* and *hfq2* might fulfill specific cellular requirements for each of the two proteins in cultures in different growth phases. Our data also suggest that, besides playing the same roles in cell physiology, Hfq and Hfq2 might have distinct functions. This is well illustrated by the increased resistance to high temperature and low pH conferred by the overexpression of Hfq in wild-type strain J2315 and by the increased resistance of the wild-type strain to osmotic and oxidative stress when overexpressing Hfq2. Both proteins also seem to play somehow distinct roles in virulence. In fact, while Hfq2 overexpression has a more pronounced effect on the rate of *C. elegans* killing, overexpression of Hfq led to higher numbers of bacterial CFU colonizing the nematode digestive tract. Nevertheless, both proteins are required for virulence, since the simultaneous inactivation of the two Hfq-like proteins results in reduced virulence compared with that of the respective single mutants. Taking these data together and considering the high level of conservation of these proteins in Bcc bacteria, results from this work suggest that although there is a partial redundancy between the functions of these two RNA chaperones, they probably have additional functions in the biology of Bcc bacteria.

Ongoing work envisages the understanding of the specific roles played by each of the two Hfq-like proteins in the expression of genes associated with the stress resistance and virulence of *B. cenocepacia* J2315.

ACKNOWLEDGMENTS

We thank the *Caenorhabditis* Genetics Center (University of Minnesota) for the kind gift of *C. elegans* DH26, Leo Eberl for *B. cenocepacia* H111 and H111-cepR, and the Coli Genetic Stock Center (Yale University) for plasmids pKD46, pKD13, and pCP20. We thank A. Azevedo and G. Gomes for help with CD experiments.

This work was supported by Fundação Ciência e Tecnologia, Portugal (contracts PTDC/BIA-MIC/65210/2006 and PTDC/EBB-BIO/098352/2008); a postdoctoral grant to S.A.S.; and a Fundação Calouste Gulbenkian doctoral grant to C.G.R.

REFERENCES

- Andrade, M. A., P. Chacón, J. J. Merelo, and F. Morán. 1993. Evaluation of secondary structure of proteins from UV circular dichroism using an unsupervised learning neural network. *Protein Eng.* **6**:383–390.
- Attia, A. S., et al. 2008. *Moraxella catarrhalis* expresses an unusual Hfq protein. *Infect. Immun.* **76**:2520–2530.
- Brahms, S., and J. Brahms. 1980. Determination of protein secondary structure in solution by vacuum ultraviolet circular dichroism. *J. Mol. Biol.* **138**:149–178.
- Brennan, R. G., and T. M. Link. 2007. Hfq structure, function and ligand binding. *Curr. Opin. Microbiol.* **10**:125–133.
- Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics* **77**:71–94.
- Casadaban, M. J. 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J. Mol. Biol.* **104**:541–555.
- Chao, Y., and J. Vogel. 2010. The role of Hfq in bacterial pathogens. *Curr. Opin. Microbiol.* **13**:24–33.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* **97**:6640–6645.
- Gottesman, S. 2004. The small RNA regulators in *Escherichia coli*: roles and mechanisms. *Annu. Rev. Microbiol.* **58**:303–328.
- Govan, J. R., and V. Deretic. 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol. Rev.* **60**:539–574.
- Govan, J. R. W., et al. 1993. Evidence for transmission of *Pseudomonas cepacia* by social contact in cystic fibrosis. *Lancet* **342**:15–19.
- Holden, M. T., et al. 2009. The genome of *Burkholderia cenocepacia* J2315, an epidemic pathogen of cystic fibrosis patients. *J. Bacteriol.* **191**:261–277.
- Kajitani, M., A. Kato, A. Wada, Y. Inokuchi, and A. Ishihama. 1994. Regulation of the *Escherichia coli* *hfq* gene encoding the host factor for phage Q_B. *J. Bacteriol.* **176**:531–534.
- Kawano, M., A. A. Reynolds, J. Miranda-Rios, and G. Storz. 2005. Detection of 5'- and 3'-UTR-derived small RNAs and cis-encoded antisense RNAs in *Escherichia coli*. *Nucleic Acids Res.* **33**:1040–1050.
- Köthe, M., et al. 2003. Killing of *Caenorhabditis elegans* by *Burkholderia cepacia* is controlled by the *cep* quorum-sensing system. *Cell. Microbiol.* **5**:343–351.
- Lagos-Quintana, M., R. Rauhut, W. Lendeckel, and T. Tuschl. 2001. Identification of novel genes coding for small expressed RNAs. *Science* **294**:853–858.
- Lefebvre, M. D., and M. A. Valvano. 2002. Construction and evaluation of plasmid vectors optimized for constitutive and regulated gene expression in *Burkholderia cepacia* complex isolates. *Appl. Environ. Microbiol.* **68**:5956–5964.
- Leitão, J. H., et al. 2010. Pathogenicity, virulence factors, and strategies to fight against *Burkholderia cepacia* complex pathogens and related species. *Appl. Microbiol. Biotechnol.* **87**:31–40.
- Link, T. M., P. Valentin-Hansen, and R. G. Brennan. 2009. Structure of *Escherichia coli* Hfq bound to polyribadenylate RNA. *Proc. Natl. Acad. Sci. U. S. A.* **106**:19292–19297.
- Meselson, M., and R. Yuan. 1968. DNA restriction enzyme from *E. coli*. *Nature* **217**:1110–1114.
- Møller, T., et al. 2002. Hfq: a bacterial Sm-like protein that mediates RNA-RNA interaction. *Mol. Cell* **9**:23–30.
- Moreira, L. M., et al. 2003. Identification and physical organization of the gene cluster involved in the biosynthesis of *Burkholderia cepacia* complex exopolysaccharide. *Biochem. Biophys. Res. Commun.* **312**:323–333.
- Moskaleva, O., et al. 2010. The structures of mutant forms of Hfq from *Pseudomonas aeruginosa* reveal the importance of the conserved His57 for the protein hexamer organization. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **66**(Pt. 7):760–764.
- Münch, R., et al. 2005. Virtual Footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. *Bioinformatics* **21**:4187–4189.
- Papenfort, K., and J. Vogel. 2010. Regulatory RNA in bacterial pathogens. *Cell Host Microbe* **8**:116–127.
- Rajkowsch, L., and R. Schroeder. 2007. Dissecting RNA chaperone activity. *RNA* **13**:2053–2060.
- Ramos, C. G., A. M. Grilo, and J. H. Leitão. 2010. Small non-coding RNAs in prokaryotes: roles in pathogenesis and potential therapeutic targets. Lambert Academic Publishing, Saarbrücken, Germany.
- Ramos, C. G., S. A. Sousa, A. M. Grilo, L. Eberl, and J. H. Leitão. 2010. The *Burkholderia cenocepacia* K56-2 pleiotropic regulator Pbr, is required for stress resistance and virulence. *Microb. Pathog.* **48**:168–177.
- Salgado-Garrido, J., E. Bragado-Nilsson, S. Kandels-Lewis, and B. Séraphin. 1999. Sm and Sm-like proteins assemble in two related complexes of deep evolutionary origin. *EMBO J.* **18**:3451–3462.
- Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schilling, D., and U. Gerischer. 2009. The *Acinetobacter baylyi* *hfq* gene encodes a large protein with an unusual C terminus. *J. Bacteriol.* **191**:5553–5562.
- Schirmer, F., S. Ehrt, and W. Hillen. 1997. Expression, inducer spectrum, domain structure, and function of MopR, the regulator of phenol degradation in *Acinetobacter calcoaceticus* NCIB8250. *J. Bacteriol.* **179**:1329–1336.
- Sousa, S. A., L. M. Moreira, and J. H. Leitão. 2008. Functional analysis of the *Burkholderia cenocepacia* J2315 BceAJ protein with phosphomannose isomerase and GDP-D-mannose pyrophosphorylase activities. *Appl. Microbiol. Biotechnol.* **80**:1015–1022.
- Sousa, S. A., et al. 2007. The *Burkholderia cepacia* *bceA* gene encodes a protein with phosphomannose isomerase and GDP-D-mannose pyrophosphorylase activities. *Biochem. Biophys. Res. Commun.* **353**:200–206.
- Sousa, S. A., C. G. Ramos, and J. H. Leitão. 2011. *Burkholderia cepacia* complex: emerging multihost pathogens equipped with a wide range of virulence factors and determinants. *Int. J. Microbiol.* **2011**:607575.
- Sousa, S. A., C. G. Ramos, L. M. Moreira, and J. H. Leitão. 2010. The *hfq*

- gene is required for stress resistance and full virulence of *Burkholderia cepacia* to the nematode *Caenorhabditis elegans*. *Microbiology* **156**:896–908.
37. Sutherland, B. W., J. Toews, and J. Kast. 2008. Utility of formaldehyde cross-linking and mass spectrometry in the study of protein-protein interactions. *J. Mass Spectrom.* **43**:699–715.
38. Updegrave, T. B., J. J. Correia, R. Galletto, W. Bujalowski, and R. M. Wartell. 2010. *E. coli* DNA associated with isolated Hfq interacts with Hfq's distal surface and C-terminal domain. *Biochim. Biophys. Acta* **1799**:588–596.
39. Valentin-Hansen, P., M. Eriksen, and C. Udesen. 2004. The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol. Microbiol.* **51**:1525–1533.
40. Vecerek, B., L. Rajkowitsch, E. Sonnleitner, R. Schroeder, and U. Blasi. 2008. The C-terminal domain of *Escherichia coli* Hfq is required for regulation. *Nucleic Acids Res.* **36**:133–143.
41. Walsh, P., D. Bursa, Y. C. Law, D. Cyr, and T. Lithgow. 2004. The J-protein family: modulating protein assembly, disassembly and translocation. *EMBO Rep.* **5**:567–571.
42. Williams, P., and M. Cámara. 2009. Quorum sensing and environmental adaptation in *Pseudomonas aeruginosa*: a tale of regulatory networks and multifunctional signal molecules. *Curr. Opin. Microbiol.* **12**:182–191.
43. Zhang, A., K. M. Wassarman, J. Ortega, A. C. Steven, and G. Storz. 2002. The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. *Mol. Cell* **9**:11–22.